

Isoform specific phosphorylation of protein phosphatase 2C expressed in COS7 cells

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Abstract Of the six distinct isoforms of mouse protein phosphatase 2C (PP2C) (α , β -1, β -2, β -3, β -4 and β -5), PP2C α was specifically phosphorylated on the serine residue(s) when expressed in COS7 cells. Analysis of phosphorylation sites using site-directed mutagenesis demonstrated that Ser-375 and/or Ser-377 were phosphorylated *in vivo*. These serine residues were the sites of phosphorylation by casein kinase II *in vitro*. Phosphorylation of PP2C α was enhanced two-fold by the addition of okadaic acid to the culture medium, but addition of cyclosporin A had no such effect. These results suggest that the expressed PP2C α is phosphorylated by a casein kinase II-like protein kinase and dephosphorylated by PP1 and/or PP2A in COS7 cells.

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Key words: Protein phosphatase 2C; Phosphorylation; Site-directed mutagenesis; Okadaic acid

1. Introduction

Protein phosphorylation plays a key role in the regulation of cellular functions, and protein phosphatases in combination with protein kinases control the phosphorylation levels of cellular proteins. The catalytic subunits of the major protein serine/threonine phosphatases found in eukaryotic cells have been divided into four classes, types 1, 2A, 2B and 2C (PP1, PP2A, PP2B and PP2C, respectively) [1]. To date the presence of five distinct PP2C genes (PP2C α , PP2C β , PP2C γ , Wip1 and FIN13) has been reported in mammalian cells [2–6]. In addition, mouse PP2C β has been found to have five distinct isoforms (β -1, β -2, β -3, β -4 and β -5) which are splicing variants originating from a single pre-mRNA [7–9]. The presence of three isoforms of human PP2C α has also been reported [10].

Recently, information on the function of mammalian PP2C has accumulated. PP2C α has been implicated in regulation of the activities of AMP-activated protein kinase and Ca²⁺/calmodulin-dependent protein kinase II, and both Wip1 and FIN13 have been found to participate in regulation of cell growth [5,6,11,12]. In this context, we have demonstrated that expression of rat PP2C α in *Saccharomyces cerevisiae* cells caused suppression of the DNA excision repair system [13]. In the same experimental system, we also found that the expressed PP2C α was phosphorylated on serine residue(s) in the yeast cells [14]. The phosphorylation site(s) was predicted to be in the carboxy-terminal region of PP2C α . However, demonstration of phosphorylation of PP2C α in mammalian cells, determination of isoform specificity of the phosphoryla-

tion and identification of the phosphorylated serine residue(s) have yet to be accomplished.

In this study, we demonstrate that of the six distinct mouse PP2C isoforms (α , β -1, β -2, β -3, β -4 and β -5) PP2C α was specifically phosphorylated on Ser-375 and/or Ser-377 in COS7 cells. We also provide evidence indicating that PP2C α is dephosphorylated by PP1 and/or PP2A in the cells.

2. Materials and methods

2.1. Materials

Glutathione agarose beads, protein A agarose beads, partially hydrolyzed α -casein and whole histone were purchased from Sigma (St. Louis, MO, USA). [γ -³²P]ATP (3000 Ci/mmol) was obtained from DuPont-New England Nuclear (Boston, MA, USA). Restriction enzymes and other modifying enzymes for DNA manipulation were from Takara (Kyoto, Japan). Anti-hemagglutinin (HA) antibody 12CA5, biotin conjugated anti-HA and CSPD were purchased from Boehringer Mannheim (Mannheim, Germany). Anti-mouse IgG-alkaline phosphatase conjugate was from Promega (Madison, WI, USA). PVDF membrane was obtained from Millipore (Bedford, MA, USA). All other reagents were from Wako Pure Chemical (Osaka, Japan).

2.2. Strains and plasmids

Escherichia coli strain BL21 was used for the expression of GST fusion proteins. Plasmids pEUK-C-1, pGEX-2T and pKF18k were purchased from Clontech Laboratories (Palo Alto, CA, USA), Pharmacia (Uppsala, Sweden) and Takara, respectively.

2.3. Construction of epitope tagged PP2C expression plasmids for COS7 cells

Expression constructs of HA epitope (YPYDVPDYA) tagged PP2C α (pEUK-C-HA-PP2C α) and of HA-PP2C β isoforms (pEUK-C-HA-PP2C β -1, -2, -3, -4 and -5) were prepared essentially as described previously [15,16].

2.4. In vitro mutagenesis

The *Eco*RI fragment (1.1 kbp) of mouse PP2C α cDNA [17] was subcloned into pKF18k. The mutagenesis was performed by the oligonucleotide-directed dual amber method [18] using the following primers: 5'-TGA TTC TGC GGC AAC CGA TGA-3' (for PP2C α -S375A), 5'-CGA CAC TGA TGC TGC GTC AAC-3' (for PP2C α -S375A) and 5'-CGA CAC TGA TGC TGC GGC AAC CGA TGA-3' (for PP2C α -S375A/S377A). The *Eco*RI fragments of pEUK-C-HA-PP2C α and pGEX-PP2C α were replaced by the mutated *Eco*RI fragments to make expression plasmids for COS7 and *E. coli* cells, respectively. The final products were termed pEUK-C-HA-PP2C α -375A (for PP2C α -S375A), pEUK-C-HA-PP2C α -377A (for PP2C α -S377A), pEUK-C-HA-PP2C α -375A/377A (for PP2C α -S375A/S377A), pGEX-375A (for PP2C α -S375A), pGEX-377A (for PP2C α -S377A) and pGEX-375A/377A (for PP2C α -S375A/S377A).

2.5. Expression of recombinant PP2C α and its mutants in *E. coli* cells

The GST-PP2C isoforms were expressed in *E. coli* BL21 and purified using glutathione agarose beads and thrombin treatment [17].

2.6. DNA sequencing

The DNA fragments generated by PCR or *in vitro* mutagenesis were examined using an ABI DNA sequencer, model 373A.

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2.7. Cell culture and transfection

The COS7 cells were grown in Dulbecco's minimal essential medium (DMEM) containing 10% (v/v) fetal calf serum, 100 units/ml penicillin G and 100 µg/ml streptomycin. The HA tagged PP2C cDNAs were transiently expressed in COS7 cells using the DEAE-dextran transfection procedure [17]. The cells were washed in ice-cold PBS 48 h after transfection and fixed with 10% (w/v) trichloroacetic acid for 10 min at 4°C. The fixed cells were collected using a cell scraper and centrifuged at 12000×g for 1 min. Sodium dodecyl sulfate (SDS) sample buffer was added to the precipitate and homogenized by sonication.

2.8. Phospholabeling of COS7 cells

The COS7 cells were washed with 150 mM NaCl and incubated with phosphate-free DMEM containing [³²P]orthophosphate (60 µCi/ml) 48 h after transfection. The dish was incubated for 5 h at 37°C and the phospholabeled cells were harvested after washing with ice-cold PBS. The cells were lysed using TNE buffer (10 mM Tris-HCl, pH 7.5, 1% (v/v) Triton X-100, 150 mM NaCl, 1 mM EDTA and 1 mM PMSF) containing 50 mM NaF and 0.1 µM okadaic acid (OA). After centrifugation, the anti-HA antibody 12CA5 was added to the supernatant and the PP2C-antibody complex was recovered using protein A agarose beads and subjected to SDS-PAGE analysis.

2.9. Phosphorylation of PP2C by casein kinase II

Casein kinase II was purified from rat liver nuclei as described by Takeda et al. [19]. The recombinant mouse PP2Cα and its point mutants were incubated with 40 mM Tris-HCl, pH 7.5, 10 mM MgCl₂, 2 mM DTT, 0.5 mM ATP (10 µCi [³²P]ATP), 0.1 mg/ml polylysine and purified casein kinase II (2 µg) in a total volume of 25 µl. The incorporated radioactivity was measured after SDS-PAGE using a BAS2000 image analyzer.

2.10. Western blot analysis

Western blotting was performed by the standard procedure, using the anti-HA antibody 12CA5 and alkaline phosphatase conjugated anti-mouse IgG. The blots were developed by chemiluminescence using CSPD as substrate. In order to demonstrate the amount of HA-PP2C protein precipitated by the anti-HA antibody, biotin conjugated anti-HA antibody and streptavidin-peroxidase conjugate was used instead of alkaline phosphatase conjugated anti-mouse IgG, because HA-PP2Cα protein migrated almost simultaneously with the heavy

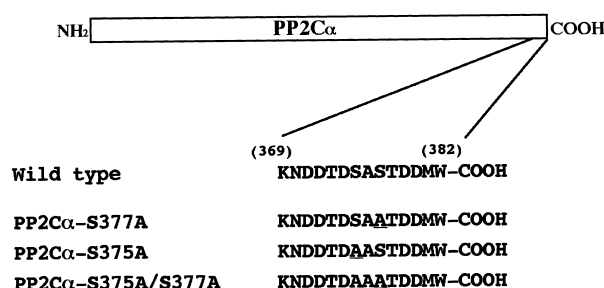


Fig. 2. Diagram of in vitro mutagenesis of PP2Cα. The mutant amino acids are underlined. The numbers indicate the positions of the amino acid residues in mouse PP2Cα.

chain of the immunoglobulin on SDS-PAGE, so that the HA-PP2Cα band was hidden by the band of the heavy chain of the IgG when anti-mouse IgG antibody was used as the secondary antibody.

2.11. Phosphoamino acid analysis

³²P-labeled PP2Cα was subjected to phosphoamino acid analysis as described by Boyle et al. [20].

2.12. Assay of protein phosphatase activity

[³²P]Phosphohistone and [³²P]phosphocasein were prepared by incubating whole histone and partially digested α-casein, respectively, with the catalytic subunit of protein kinase A and [³²P]ATP as described previously [21,22]. The catalytic subunit of protein kinase A was purified as described by Reimann and Beham [23]. Protein phosphatase activity was assayed by measuring the release of [³²P]phosphate from the [³²P]phosphohistone or [³²P]phosphocasein, essentially as described previously [21]. One unit of activity was defined as the amount of enzyme which catalyzes the release of 1 nmol phosphate per min.

3. Results

3.1. Phosphorylation of HA-PP2C isoforms expressed in COS7 cells

Cell extracts from COS7 cells transfected with expression plasmids for six distinct mouse PP2C isoforms (α, β-1, β-2, β-3, β-4 and β-5) were analyzed by Western blotting using the anti-HA monoclonal antibody (12CA5). Protein bands of 46–48 kDa were observed only when the cells were transfected with the expression plasmids (Fig. 1A, lower panel). In order to determine whether the HA tagged PP2C isoforms exhibited the protein phosphatase activities characteristic of PP2C, the activities of the immunoprecipitates were measured using [³²P]phosphohistone phosphorylated by protein kinase A as the substrate. All the immunoprecipitates from the cell extracts that had been transfected with the expression vectors showed substantial levels of Mg²⁺ dependent and OA insensitive histone phosphatase activity (data not shown). However, no protein phosphatase activity was detected in immunoprecipitates from cells transfected only with the control vector plasmid.

In order to test whether PP2C isoforms were phosphorylated when expressed in mammalian cells, we performed phospholabeling of COS7 cells expressing HA tagged PP2C proteins. COS7 cells transfected with the expression plasmids were metabolically labeled with [³²P]orthophosphate and lysed using a buffer containing Triton X-100. The PP2C isoforms were immunoprecipitated with the anti-HA antibody and analyzed by SDS-PAGE, and the radioactivities in the gel were visualized by autoradiography. As shown in Fig.

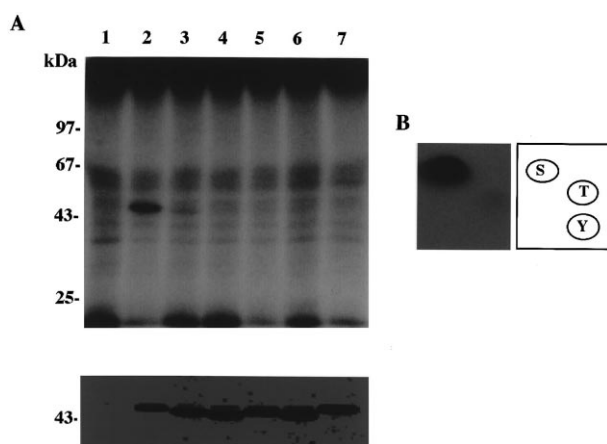


Fig. 1. Phosphorylation of HA tagged PP2C isoforms in COS7 cells. A: Upper panel: COS7 cells transfected with pEUK-C-1 (lane 1), pEUK-C-HA-PP2Cα (lane 2), pEUK-C-HA-PP2Cβ-1 (lane 3), pEUK-C-HA-PP2Cβ-2 (lane 4), pEUK-C-HA-PP2Cβ-3 (lane 5), pEUK-C-HA-PP2Cβ-4 (lane 6) and pEUK-C-HA-PP2Cβ-5 (lane 7) were phospholabeled by adding [³²P]orthophosphate to the culture medium. The cell extracts were immunoprecipitated by the anti-HA antibody and the immunoprecipitates were subjected to SDS-PAGE. The results of autoradiography are shown. Lower panel: Western blot analysis was performed using the anti-HA antibody. B: The protein band for phospho-HA-PP2Cα (as shown in A) was excised from the gel and subjected to phosphoamino acid analysis.

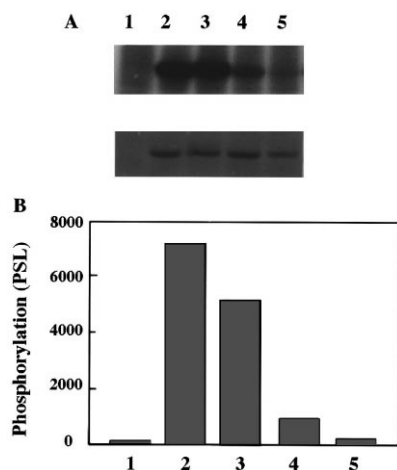


Fig. 3. Phosphorylation of mutant PP2C α proteins by casein kinase II. A: Upper panel: Wild-type and mutant PP2C α were expressed in *E. coli* cells, purified by glutathione agarose beads and thrombin treatment, and phosphorylated by casein kinase II. The results of autoradiography after SDS-PAGE are shown. Lower panel: The gel was stained with Coomassie brilliant blue after SDS-PAGE. B: The radioactivities incorporated into the PP2C α proteins (A) were determined using a BAS 2000 image analyzer. Lane 1, no PP2C protein; lane 2, PP2C α ; lane 3, PP2C α -S377A; lane 4, PP2C α -S375A; lane 5, PP2C α -S375A/S377A.

1A, PP2C α , but no other isoform, was phosphorylated in the cells. Phosphoamino acid analysis using high voltage thin layer chromatography demonstrated that phosphorylation of PP2C α occurred almost exclusively on serine residues (Fig. 1B). A trace amount of threonine phosphorylation was observed, but no tyrosine phosphorylation could be detected.

3.2. Phosphorylation of mutant PP2C α in vitro and in vivo

The putative phosphorylation sites of rat PP2C α suggested by our previous study were in the carboxy-terminal region, which contained two serine residues (Ser-375 and Ser-377) [14]. The sequence of the carboxy-terminus including Ser-375 and Ser-377 was conserved in mouse PP2C α (Fig. 2) [17]. In order to determine the actual phosphorylation sites, we introduced a mutation from serine to alanine at these two serine sites by site-directed mutagenesis (Fig. 2). Fragments of mutant PP2C α were introduced into either the pGEX-2T vector (for GST fusion) or pEUK-C vector (for COS7 expression). All three mutants were expressed as effectively as wild-type PP2C α in both *E. coli* (Fig. 3A, lower panel) and COS7 cells (Fig. 4A, lower panel).

The recombinant PP2C α mutant proteins were incubated with casein kinase II and [γ - 32 P]ATP (Fig. 3A,B). The wild-type mouse PP2C α was phosphorylated by casein kinase II, confirming our previous observation with rat PP2C α [14]. The stoichiometry of the phosphorylation was 1.2 mol phosphate per mol protein. The Ser-375 \rightarrow Ala-375 mutation (PP2C α -S375A) caused an 87% decrease in the phosphorylation of PP2C α (lane 4). However, the decrease in phosphorylation produced by the Ser-377 \rightarrow Ala-377 mutation (PP2C α -S377A) was only 28% compared with the control (lane 3). The mutant in which both serine residues were changed to alanine (PP2C α -S375A/S377A) was not phosphorylated (lane 5). These results indicate that the major phosphorylation

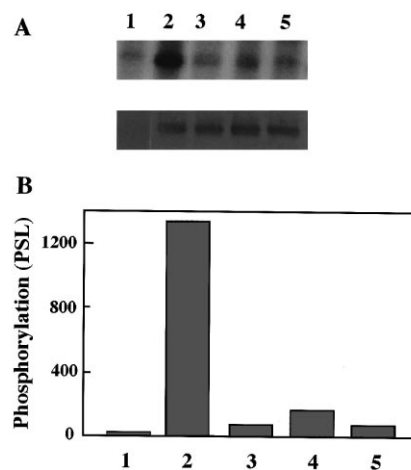


Fig. 4. Phosphorylation of mutant HA tagged PP2C α proteins in COS7 cells. A: Upper panel: Wild-type and mutant HA-PP2C α proteins were transiently expressed in COS7 cells and metabolically labeled by [32 P]orthophosphate. The labeled proteins were immunoprecipitated by the anti-HA antibody and subjected to SDS-PAGE. The radioactivities incorporated into the PP2C α proteins were visualized by autoradiography. Lower panel: Western blot analysis was performed using the anti-HA antibody. B: The radioactivities incorporated into the PP2C α proteins were determined using a BAS 2000 image analyzer. Lane 1, pEUK-C-1; lane 2, pEUK-C-HA-PP2C α ; lane 3, pEUK-C-HA-PP2C α -377A; lane 4, pEUK-C-HA-PP2C α -375A; lane 5, pEUK-C-HA-PP2C α -375A/377A.

site for casein kinase II is Ser-375. Ser-377 was also phosphorylated by casein kinase II but to a much smaller extent.

Fig. 4 shows the results of the in vivo experiments. As in the in vitro experiments, the double mutant HA-PP2C α -S375A/S377A was not phosphorylated when expressed in COS7 cells (lane 5), indicating that at least one of these two serines was phosphorylated in COS7 cells. However, in contrast to the in vitro experiments, essentially no phosphoryla-

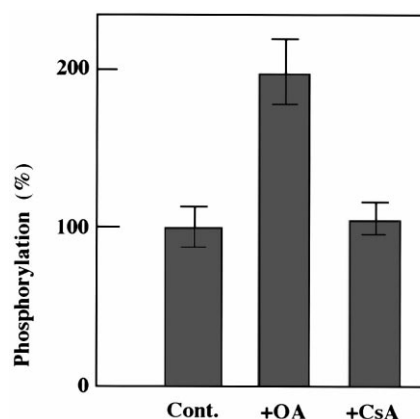


Fig. 5. Effects of phosphatase inhibitors on the phosphorylation of HA tagged PP2C α in COS7 cells. COS7 cells harboring the expression vector for HA-PP2C α were labeled by [32 P]inorganic phosphate. OA (1 μ M) or CsA (1 μ g/ml) was added to the phospholabeling medium 1 h before harvesting. The cells were then collected and lysed. The HA-PP2C α proteins were immunoprecipitated from the cell extracts by the anti-HA antibody. After SDS-PAGE of the immunoprecipitates, the radioactivities incorporated into the PP2C α proteins were determined using a BAS 2000 image analyzer. The results represent the means of three separate experiments \pm S.D.

tion was observed in HA-PP2C α -S377A (lane 3) while HA-PP2C α -S375A (lane 4) was slightly phosphorylated in COS7 cells.

3.3. Effect of phosphatase inhibitors on the phosphorylation of PP2C α

We next determined whether PP2C α phosphorylated in COS7 cells was dephosphorylated by endogenous PPs. To this end we tested the effects of protein phosphatase inhibitors on the phosphorylation of PP2C α expressed in COS7 cells. Following incubation of the COS7 cells with [³²P]orthophosphate, 1 μ M OA or 1 μ g/ml cyclosporin A (CsA) was added to the labeling mixture and incubated for another 1 h. The labeled PP2C α was immunoprecipitated with the anti-HA antibody and the level of phosphorylation was determined using a BAS 2000 image analyzer after SDS-PAGE (Fig. 5). The phosphorylation of PP2C α was increased two-fold by the addition of OA. However, the addition of CsA did not affect phosphorylation. These results indicated that endogenous PPs responsible for the dephosphorylation of PP2C α in COS7 cells were PP1 and/or PP2A.

4. Discussion

In our previous study, we have demonstrated that rat PP2C α expressed in *S. cerevisiae* cells was phosphorylated on serine residues of its carboxy-terminal region [14]. However, whether the phosphorylation was a physiological phenomenon was not known since the experiments were performed using a heterologous system, namely expression of mammalian PP2C in yeast cells. In addition, the putative phosphorylation sites of rat PP2C α were not conserved in the *S. cerevisiae* PP2Cs. In this study, we demonstrated for the first time the isoform specific phosphorylation of mammalian PP2C in mammalian cells, indicating that the phosphorylation of PP2C α is a physiologically occurring event.

Since the double mutant of PP2C α (S375A/S377A) was not phosphorylated both in vivo and in vitro (Figs. 3 and 4), it was suggested that PP2C α was phosphorylated by casein kinase II or casein kinase II-like protein kinase in COS7 cells. However, a marked difference was observed in the phosphorylation level of Ser-375 between in vivo and in vitro experiments when phosphorylation of a single mutant (S377A) was examined. The reason for this discrepancy is not known. One unique feature of the in vivo condition is that endogenous PP(s) may affect the phosphorylation of recombinant PP2C α . It was indicated that PP1 and/or PP2A are responsible for the dephosphorylation of PP2C α in COS7 cell. Therefore, it seems reasonable to speculate that the difference in the phosphorylation level between the in vivo and in vitro experiments was caused by a higher susceptibility of phosphoserine-375 in the single mutant S377A to endogenous PP1 and/or PP2A than that in wild-type PP2C α . This may have been caused by the absence of phosphoserine-377 in the mutant molecule. Alternatively, phosphorylation of Ser-377 may be a prerequisite for the phosphorylation of Ser-375 in vivo.

Peruski et al. isolated the human cDNAs encoding three isoforms of PP2C α (α 1, α 2 and α 3) by RT-PCR from HL60 monocytes [10]. They reported that PP2C α 1 was the dominant isoform, comprising 75% of the PP2C α cDNAs generated, and that the amounts of PP2C α 2 and PP2C α 3 were relatively low. The deduced amino acid sequences indi-

cated that the three isoforms were similar but a significant difference was observed at their carboxy-terminal regions. Of these three human PP2C α isoforms PP2C α 1 was most homologous to rat and mouse PP2C α . Interestingly, the two serines corresponding to Ser-375 and Ser-377 of mouse PP2C α are conserved in human PP2C α 1 and PP2C α 3 but not in PP2C α 2, in which the amino-terminal side Ser is changed to Tyr. Therefore, human PP2C α 1 and PP2C α 3 but not PP2C α 2 may also be phosphorylated in vivo.

In our previous study, we demonstrated that the phosphorylation by casein kinase II did not affect the enzyme activity of PP2C α as far as artificial substrates (phosphohistone or phosphocasein) were used in the enzyme assay. However, it still seems possible that the activity against physiological substrate(s) is regulated by the phosphorylation. Alternatively, the phosphorylation may affect the binding of PP2C α to other proteins in the cells or it may regulate the subcellular localization of PP2C α . Studies to verify these hypotheses are under way now.

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